PATENT
Attorney Docket No. **PRMG 04578**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Slater, et al.	Group No.:	1652
Serial No.:	09/641,319	Examiner:	R. Hutson
Filed:	08/18/2000		
Entitled:	**Thermophilic DNA Polymerases From Thermotoga Neapolitana**		

DECLARATION OF JAMES HARTNETT UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING UNDER 37 C.F.R. 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 11/12/2003 By: Susan M. McClintock

Examiner Hutson:

I, James Hartnett, hereby declare and state, under penalty of perjury, that:

1. I am one of the inventors of the above-named patent application (hereinafter "present application").

2. The claimed invention was reduced to practice in the United States of America prior to January 9, 1995, as indicated from this Declaration and the attached notebook pages. The work presented in the notebook pages was performed in this country by me or under my supervision. As evidenced from these documents, we developed mutant DNA polymerases derived from *Thermotoga neapolitana*. This includes mutants shown in Figure 4 of the present application. For example, the deletion mutant *Thermotoga neapolitana* polymerase labeled as M284, which provides a reduction in 5'-3' exonuclease activity through generation of deletion mutations is described on pages 32 (design) and 50 (activity assay of generated mutant) of the notebook 100893. Likewise, substitution mutants that reduce 3'-5' exonuclease activity were generated. For example, the combination deletion and substitution mutant *Thermotoga*

neapolitana polymerase labeled as M284 (D323A, D389A) in Figure 4 of the present application is described on pages 27 (design) and 54 (use of the mutant enzyme in sequencing reaction) of notebook 100996 (the D323A is referred to as D355A and D389A is referred to as D424A in the notebook).

3. I further declare that all statements made herein are of my own knowledge, are true, and that all statements are made on information and belief that are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application of any patent issued thereon.

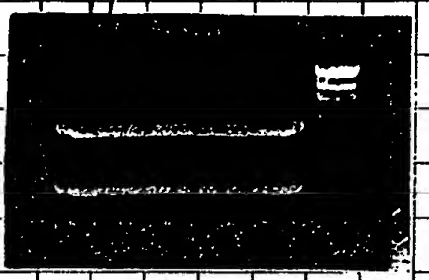
Dated: 11-12-03

James Hartnett

To remove the 5'→3' exo activity of Tne, one course of action will be to digest the gene at Met 284 with BspHI and ligate that to the NcoI site of pALTERex1. This removes 283 AA's from the N-terminal end. The BspHI site isn't unique so I have to do some monkeying around.

Digest 1

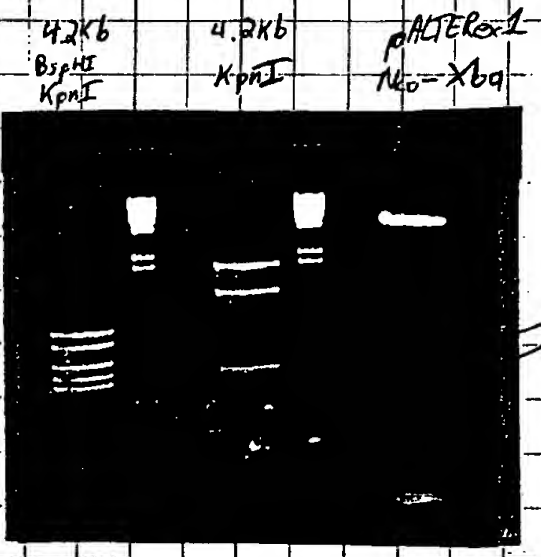
10µl pGTne 1
2µl XbaI
2µl NdeI
Buffer D



4.2 kb fragment removed and purified with Wizard DNA clean-up.

Digest 2

25µl	4.2 kb fragment		17µl	4.2 kb fragment
3µl	BspHI NEB		2µl	KpnI
2µl	KpnI		5µl	Buffer J
5µl	Buffer (4) NEB			



All fragments seen are of the expected size.
The 1050 bp BspHI – KpnI pol fragment
1300 bp KpnI – XbaI pol fragment
5846 bp NcoI – XbaI vector

were co-isolated with Wizard and ligated in a 50µl volume.

Continued on Page 34

Read and Understood By

Jim Hartnett — Signed
REDACTED — Date
Claire Johnson — Signed
REDACTED — Date

PROJECT 5'→3' exo assay

Notebook No. 49

Continued Fr m Pag

The 5'→3' exo assay consists of incubating two ^{32}P labeled substrate with 5u of enzyme for one hour at 74°C and then looking for ^{32}P release off the substrates. Reactions done in duplicate. Release measured by DE81 binding.

Results

We expected Taq and Tne (native) demonstrate a 5'→3' exo activity, whereas UlTma and M284 Tne do not. I don't feel that the 2.7% release from M284 is significant since only one of the two duplicates is showing release.

	SAMPLE	CPM1	
Ø No enzyme	1	3880.00	
	2	3978.00	
Ø	3	4016.00	
	4	3966.00	7% release
Taq	5	3808.00	
	6	3582.00	7.4%
UlTma	7	4076.00	
	8	3842.00	0%
Tne	9	3478.00	
	10	3552.00	11.9%
M284	11	3998.00	
	12	3768.00	2.7%
Ø	13	3888.00	
	14	4012.00	
	15	3758.00	
	16	3948.00	7% release
Taq	17	1170.00	
	18	1132.00	70%
UlTma	19	4060.00	
	20	4044.00	0%
Tne	21	2770.00	
	22	2738.00	28%
M284	23	4074.00	
	24	3992.00	0%

Continued on Page 51

Read and Understood By

Jim Hartsough — Signed

REDACTED — Date

Elaine Ackerman — Signed

REDACTED — Date

To be sure 3'→5' exo activity is completely removed I want to have a D355A, D424A double mutant. This is accopliched by ~~transferring the~~ exchanging 215 bp. Csp45I fragment of D355A into the D424A background.

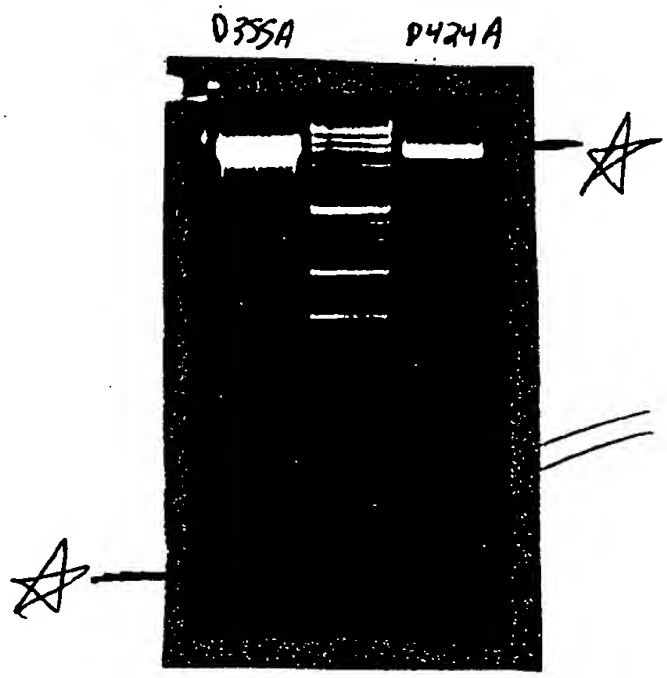
4 µg D355A plasmid cut with Csp45I
1 µg D424A plasmid cut with Csp45I

D424A reaction phosphatased
run on 1.5% TAE gel
indicated bands co-isolated with Wizard
Ligated in 40µl volume overnight at 16°C

10-18-94 Ligations transformed into JM109

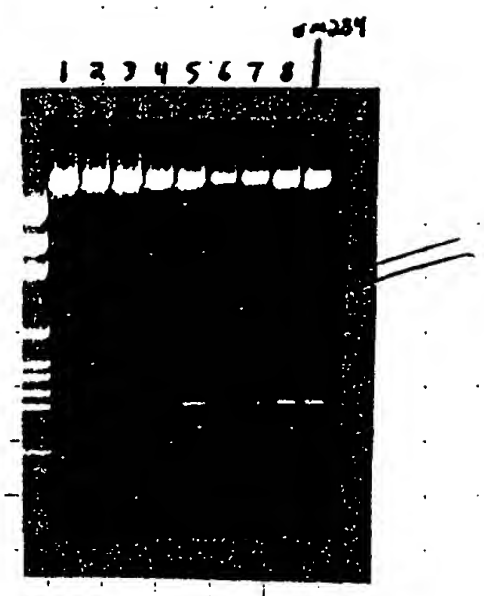
10-19-94 8 colonies streaked out

10-20-94 8 mini preps done and cut with BglII



Results

⑤, ⑥, ⑦, +⑧ show the expected 348bp. BglII fragment, although ⑥ seems to have an extra band.



Continued on Page 28

Read and Understood By

Signed	Date	Signed	Date
Jim Hartsell	REDACTED	Claire Ackerman	REDACTED

I used the following dd NTPs
Template was Tne mutant 75-5A1 and
primer was JH66. 70°↔95°.

G = 30 μM
A = 75 μM
T = 75 μM
C = 30 μM

Results

Tne doesn't look much better.
Taq looks very unbalanced.
I need to repeat this + try
lower dd NTPs for A, T, + C.

Continued on Page 56

Read and Understood By

Signed	Date	Signed	Date
[signature]	REDACTED	[signature]	REDACTED